STANDARD OPERATING PROCEDURE FOR THE DETERMINATION OF ACRYLAMIDE USING LC-MS/MS ON AB SCIEX Q TRAP 4000

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1.0 Disclaimer

This standard operating procedure has been prepared for use of the Environmental Sciences Division, Environmental Chemistry Branch, NERL, ORD of the U.S. Environmental Protection Agency and may not be specifically applicable to the activities of other organizations. **THIS IS NOT AN OFFICIAL EPA APPROVED METHOD.** This document has not been through the Agency's peer review process or ORD clearance process. Additionally, this SOP is equipment and/or instrument-specific.

2.0 Purpose (Scope and Application)

This document describes the procedure for the determination of acrylamide (AA) in aqueous samples by solid-phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an AB Sciex 4000 Q Trap MS in the triple quadrupole mode.

3.0 Method Summary

3.1 The method employs high-performance liquid chromatography (HPLC) coupled with positive electrospray ionization (ESI+) tandem mass spectrometry (MS/MS) for the determination of AA in aqueous matrices.

3.2 Aqueous samples are first flowed through activated carbon SPE cartridges to extract the acrylamide from solution before concentrating the samples to 0.5 mL.

3.3 Target compounds are identified by retention time and multiple-reaction monitoring (MRM) transitions using MS/MS. Compounds are quantified using isotope dilution.

4.0 Interferences

4.1 Method interferences can be caused by contaminants in glassware, solvents, and other apparatus, producing discrete artifacts or elevated baselines. These materials are routinely demonstrated to be free from interferences by analyzing laboratory reagent blanks and method blanks under the same conditions as the samples.

4.2 Matrix interferences may be caused by contaminants that are co-extracted from the sample.

5.0 Safety

5.1 All of the chemicals used in this procedure should be handled only while using proper personal protective equipment such as gloves, lab coats, safety glasses and fume hoods. The analyst should review the Material Safety Data Sheet for each chemical in this procedure so that safe working conditions can be achieved.

5.2 The toxicity of each reagent used in this method may not have been fully established. Each chemical should be regarded as a potential health hazard, and exposure should be kept as low as reasonably achievable. AA is a potential carcinogen and neurotoxin (http://www.epa.gov/iris/subst/0286.htm).

5.3 Waste must be disposed of in appropriate waste containers. Contact the onsite SHEM Program Manager to dispose of full waste containers.

5.4 Exhaust fumes from the HPLC-MS must be properly vented.

5.5 All applicable safety and compliance guidelines set forth by the EPA and by federal, state, and local regulations must be followed during the performance of this SOP. Stop all work in the event of a known or potential compromise to the health and safety of any person and immediately notify the SHEM Program Manager and other appropriate personnel.

5.6 Analysts must be cognizant of all instrumental hazards (i.e., dangers from electrical shock, heat, or explosion).

6.0 Reagents/Chemicals/Gases

6.1 HPLC-gradc Mcthanol

6.2 Deionized (DI) water: in-house 18 MΩ-cm DI water

6.3 AA standard (CAS# 79-06-1)

6.4 Deuterated AA (AA-d_3) standard (CAS# 122775-19-3)

6.5 ^{13}C-labeled AA (AA-$^{13}C_1$) standard (CAS # 287399-24-0)

6.6 Formic Acid

6.7 Hydroquinone (added to stock AA standards at concentration of 100 ppm to prevent polymerization of AA).

6.8 Acetonitrile

6.9 Polypropylene glycol standard from AB SCIEX

6.10 Source of pure nitrogen, either PEAK nitrogen generator, ultra-high-purity grade compressed nitrogen, and/or 230 L tank of liquid nitrogen able to output gas greater than 100 psi.

7.0 Equipment and Supplies

7.1 HPLC-MS/MS system: Shimadzu liquid chromatograph system consisting of LC-20AD pumps, an SIL-20AC HT autosampler, and a CTO-20A column oven. Detection performed using an AB Sciex 4000 Q Trap MS in the triple quadrupole mode. The MS is equipped with a Turbo V Ion Source, which utilizes the TIS source probe for positive-mode electrospray ionization (ESI+). Other HPLC-MS/MS instrumentation may be used provided that appropriate instrument parameters are optimized and sensitivity is comparable or improved.

7.2 Data acquisition/analysis software: Analyst version 1.5.2, firmware version of at least M401402 B4T0301 M3L1317 B3T0300. GPIB 2.5 driver for Analyst Software.

7.3 HPLC column (Dionex IonPac ICE-AS1 250 x 4 mm, 7.5 µm). Other columns may be used if they provide sufficient retention and separation of the target analytes; however, acrylamide is not retained by traditional HPLC columns. The IonPac ICE-AS1 columns separate according to ion exclusion chromatography. Other potential columns that might retain AA are those based on hydrophilic interaction chromatography (HILIC), but these have not been investigated.

7.4 Variable volume standard pipettors (0.5 -10 µL, 20-200 µL, 100-1000 µL)

7.5 Pipet tips

7.6 Glass beakers, volumetric flasks, sized as appropriate

7.7 Disposable borosilicate Pasteur pipets

7.8 1 mL autosampler vials with PTFE/silicone septa

7.9 Disposable 0.45 µm syringe tip filters, if needed to remove suspended solids

7.10 Filtering apparatus for filtering large volume samples using glass fiber filter discs

(type 934-AH, or equivalent), if necessary

7.11 TurboVap Concetrator, for concentrating samples

7.12 Autotrace SPE Workstation

7.13 Activated carbon SPE cartridges (Biotage, 500 mg, 6 cc size)

8.0 Sample Collection, Preservation, and Storage

8.1 This SOP does not describe sample collection procedures; however, the following guidelines are followed once samples are received in the laboratory.

8.2 Samples must be stored at 4°C in a designated sample refrigerator.

8.3 Holding time studies have not been performed on these analytes; however, samples should be analyzed as soon as possible, and within 14 days.

9.0 Quality Control

9.1 The following are relevant QC criteria for this method.

Table 1. Data Quality Indicators of Measurement Data.

QC Check	Frequency	Completeness	Precision	Accuracy	Corrective Action
Initial 5-point calibration	Prior to sample analysis	100%	RSD≤20%	$R^2 > 0.99$	No samples will be run until calibration passes criteria.
Laboratory blank	One per batch of samples[a]	100%	N/A	< PQL[b]	Inspect the system and reanalyze the blank. Samples must be bracketed by acceptable QC or they will be invalidated.
Instrument blank	One at beginning of each 8-hr analytical day, one at beginning of each batch of samples[a], and one at end of analytical day	100%	N/A	< PQL[b]	Inspect the system and reanalyze the blank. Samples must be bracketed by acceptable QC or they will be invalidated.
Laboratory fortified matrix	One per batch of samples[a]	100%	RPD≤30%[c]	70-130% recovery	Review data to determine whether matrix interference is present. If so, narrate interference and flag recovery. If no interference is evident, verify the instrument is functioning properly by running a lab blank. Reanalyze recollected sample to verify recovery. Samples must be bracketed by acceptable QC or they will be invalidated.

Laboratory replicates	One per batch of samples[a]	100%	RPD≤30%[c]	N/A	Inspect the system, narrate discrepancy. Samples must be bracketed by acceptable QC or they will be invalidated.
Continuing calibration verification (CCV)	One at beginning of each 8-hr analytical day, one at beginning of each batch of samples[a], and one at end of analytical day	100%	RSD≤30%[c]	± 30% of known value	Inspect system and perform maintenance as needed. If system still fails CCV, perform a new 5-point calibration curve. Samples must be bracketed by acceptable QC or they will be invalidated.
Laboratory fortified blank	One per batch of samples[a]	100%	RPD≤30%[c]	70-130% recovery	Inspect the system and reanalyze the standard. Re-prepare the standard if necessary. Re-calibrate the instrument if the criteria cannot be met. Samples must be bracketed by acceptable QC or they will be invalidated.
Minimum detection limit	Each chemical	100%	TBD for each HF chemical	TBD for each HF chemical	TBD for each HF chemical

[a]Batch of samples not to exceed 20
[b]PQL=practical quantitation limit, 5 times the MDL
[c]Precision among replicates if more that 1 batch of samples are analyzed. RSD may be applicable if more than 2 replicates are analyzed.

9.2 The QC checks described in Table 3 are defined here:

CALIBRATION CURVE: Prepared from calibration standards (and internal standards, if applicable) at a minimum of 5 concentrations, used to calibrate the instrument response with respect to analyte concentration.

CALIBRATION STANDARD: A solution of the target analytes prepared from the primary dilution standard solution(s) or stock standard solution(s) and internal standards.

CONTINUING CALIBRATION VERIFICATION: A calibration standard containing the method analytes and internal standards that is analyzed periodically to verify the accuracy of the existing calibration.

INSTRUMENT BLANK: A blank matrix that is identical to the matrix the analytes are analyzed in, and is analyzed periodically to determine if the method analytes have contaminated the instrument used for analysis.

LABORATORY BLANK: An aliquot of reagent water or other blank matrix that is treated exactly as a sample, including exposure to all storage containers, buffers, preservatives, and internal standards. The laboratory blank is used to determine if the method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

LABORATORY REPLICATE: A minimum of two sample aliquots taken in the laboratory from a single sample bottle and analyzed separately with identical procedures.

Analyses of replicates indicate precision associated specifically with the laboratory procedures by removing variation contributed from sample collection, preservation, and storage procedures.

LABORATORY FORTIFIED BLANK: An aliquot of reagent water or other blank matrix to which a known quantity of the method analytes is added. The laboratory fortified blank is analyzed exactly like a sample, including any applicable preservation procedures. Its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate measurements.

LABORATORY FORTIFIED MATRIX: An aliquot of a sample to which a known quantity of the method analytes is added. The laboratory fortified matrix is processed and analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentration of the analytes in the sample matrix must be determined in a separate aliquot, and the measured value in the laboratory fortified matrix corrected for background concentrations.

METHOD DETECTION LIMIT: The minimum concentration of an analyte that can be identified, measured and reported with 99% confidence that the analyte concentration is greater than zero. This is a statistical determination according to 40 CFR Part 136, Appendix B, and accurate quantitation is not expected at this level.

10.0 Calibration and Standardization

10.1 Tune and calibrate MS (both Q1 and Q3) according to manufacturer's directions using the AB Sciex PPG standard (diluted 50:1 with 50/50 water/methanol containing 0.1% formic acid and 2 mM ammonium acetate).

10.2 Tuning to determine the correct system settings (e.g., curtain gas, temperature, IonSpray voltage, declustering potential, etc.) for particular analytes is performed as needed and according to the manufacturer's directions. This is done according to the manufacturer's instructions either manually or using the Compound Optimization feature in the Analyst software. Representative settings for the analytes in this method are listed in section 11.4.

10.3 Record all instrument maintenance in the instrument maintenance log book.

10.4 Calibration standards are prepared in methanol using appropriate volumetric glassware. Stock calibration standards must be replaced at a minimum frequency of every 6 months if not previously discarded.

10.5 Suggested concentrations for the initial AA calibration levels are 10 to 800 ppb AA, each containing a constant concentration of 200 ppb AA-d_3 and 200 ppb AA-$^{13}C_1$. A minimum of 5 calibration levels must be used. Example concentrations are provided in Table 2.

Table 2. Concentrations of Calibration Standards (ppb)

Calibration Level	AA-$^{13}C_1$	AA-d_3	AA
1	200	200	10
2	200	200	20
3	200	200	50
4	200	200	100
5	200	200	200
6	200	200	300
7	200	200	500
8	200	200	800

10.6 Calibration by isotope dilution: isotope dilution is used for calibration of each native compound for which a labeled analog is available.

10.6.1 To calibrate the system by isotope dilution, inject the calibration standards (minimum of 5).

10.6.2 For the compounds determined by isotope dilution, the relative response (RR) (labeled to native) vs. concentration in the calibration solutions is computed over the calibration range according to the procedure below. Determine the response of each compound relative to its labeled analog using the area responses of the fragment ions specified in section 11.4.

10.6.3 Calibrate the native compounds with a labeled analog using the following equation:

$$44\ L\ \frac{\text{¤}_{\text{Ù}}\text{¼}_{\text{x}}}{\text{¤}_{\text{x}}\text{¼}_{\text{Ù}}}$$

Where:

A_n= Area of fragment ion of native compound (AA)
A_l= Area of fragment ion of labeled compound (AA-d_3)
C_l= Concentration of labeled compound (AA-d_3) in the calibration standard
C_n=Concentration of native compound (AA) in the calibration standard.

10.6.4 Compute the average (mean) RR, and the standard deviation and relative standard deviation (RSD) of the RRs.

10.6.5 Linearity: If the RR for any compound is constant (less than 20% RSD), the average RR may be used for that compound; otherwise, the complete calibration curve for that compound must be used over the calibration range.

10.7 Calibration by internal standard is applied to the determination of the labeled compound (AA-d_3) for determination of the extraction recovery. The AA-$^{13}C_1$ is used as the reference compound for AA-d_3. Calibration is performed at a single concentration of the AA-d3 using data from the calibration curve (Section 10.5, Table 2).

10.7.1 Response factors: internal standard calibration requires the determination of response factors (RF) defined by the following equation:

$$4(\mathbf{L} \frac{\text{¤}_{\text{Ù}}\text{¼}_{\text{ûÞ}}}{\text{¤}_{\text{ûÞ}}\text{¼}_{\text{ÙÙ}}}$$

Where:

A_n= Area of fragment ion of AA-d_3
A_{is}= Area of fragment ion of internal standard (AA-$^{13}C_1$)
C_{is}= Concentration of internal standard (AA-$^{13}C_1$) in the calibration standard.
C_n=Concentration of AA-d_3 in the calibration standard.

10.7.2 To calibrate the analytical system for AA-d_3, use the data from the calibration curve (Section 10.5 and Table 2).

10.7.3 Compute the RF for AA-d_3 using AA-$^{13}C_1$.

10.7.4 Linearity: If the RF for AA-d_3 is constant (less than 35% RSD), the average RF may be used for AA-d_3; otherwise, the complete calibration curve for AA-d_3 must be used over the calibration range.

11.0 Procedure

11.1 Glassware cleaning

11.1.1 Prepare soapy bath with hot water and approximately 1 tsp Alconox detergent. Scrub glassware with bottle brushes and/or pipe cleaners until visibly clean (do not scratch glassware with metal from brushes). Rinse glassware first with non-DI water, and then with DI water. Soak glassware in acid bath (3 mL HCl, 3 mL HNO_3, 4 L water, pH 1-2) overnight. Remove glassware and rinse with Ultrapure DI water. Rinse glassware

with methanol and air dry. Place glassware in oven at 100°C for 6 hours.

11.2 Sample preparation

11.2.1 Add an appropriate amount of deuterated AA (AA-d3) to a known volume of aqueous sample for extraction. For the purposes of this method, the addition of 100 ng of AA-d3 to 500 mL sample worked well.

11.2.2 If aqueous sample contains suspended solids, first filter using filtering apparatus and glass fiber discs. Ensure that the glass fiber disc is rinsed well with water, followed by MTBE.

11.3 Solid-phase extraction

11.3.1 Load cartridges into Autotrace SPE Workstation, and condition the cartridges with 5 mL methanol, followed by 5 mL water, both at a flow rate of 5 mL/min.

11.3.2 Load 500 mL aqueous sample through the SPE cartridges at a flow rate of 5 mL/min. Rinse the volumetric flasks with 50 mL water, and load this water through the SPE cartridges as well.

11.3.3 Rinse the cartridges with 2 mL water at a flow rate of 3 mL/min, and dry the cartridges with N_2 for 30 min.

11.3.4 Elute with 10 mL methanol at a flow rate of 1 mL/min.

11.3.5 Quantitatively transfer the eluate from the Autotrace collection tube to a TurboVap tube. Concentrate the eluate with methanol using the TurboVap Concentrator. Concentrate to 0.5 mL in methanol.

11.3.6 Transfer the concentrated sample with Pasteur pipet to an autosampler vial.

11.3.7 Add 10 µL of 10 ng/µL AA-$^{13}C_1$ to each sample

11.3.8 Filter the samples, if necessary, with a syringe filter prior to LC-MS/MS analysis.

11.4 LC-MS/MS analysis

11.4.1 The LC and MS/MS parameters described in this section for the analysis of AA are saved in the method "AA_20120621.dam".

11.4.2 Mobile phase A consists of 0.1% FA in water. Mobile phase B consists of 0.1% FA in acetonitrile.

11.4.3 The chromatographic conditions include a 30-minute isocratic elution off the Dionex IonPac column at 50% B at a flow rate of 0.160 mL/min (column pressure not to exceed 1000 psi, column temperature of 30°C). Use an injection volume of 30 µL.

11.4.4 MS source conditions: Curtain gas: 40 psi, IonSpray Voltage: 5500 V, Temperature: 400°C, Ion Source Gas 1 (nebulizer gas): 40 psi, Ion Source Gas 2 (auxiliary gas): 60 psi, Collision Gas: 5, Interface Heater: On, Needle position: x=5.0 mm, y= 2.5 mm.

11.4.5 MRM settings are provided in Table 3.

Table 3. Table of MRM parameters for AA native and labeled compounds.

Compound	Precursor m/z	Fragment m/z	DT[a] (msec)	DP[b] (V)	EP[c] (V)	CE[d] (V)	CXP[e] (V)
AA	72.1	55.1	150	51	5	17	10
AA-d_3	75.1	58.0	150	51	5	17	10
AA-$^{13}C_1$	73.1	56.0	150	51	5	19	10
AA (conf.)	72.1	44.1	150	51	5	25	6
AA-d_3 (conf.)	75.1	44.1	150	51	5	31	6
AA-$^{13}C_1$ (conf.)	73.1	45.1	150	51	5	27	6

[a]DT = dwell time
[b]DP = declustering potential
[c]EP = entrance potential
[d]CE = collision energy
[e]CXP = collision cell exit potential

11.4.6 Precursor m/z values in Table 3 are the $[M+H]^+$ ions. The monitored quantitative MRM transition in the MS/MS experiments for each compound involves a loss of NH_3. The MRM transition for the confirmation ion involves a loss of C_2H_4.

11.4.7 To run samples: Log into computer. Username = ABSystems, password = ABSystems. If not already open, double-click on icon for Analyst 1.5.2. Ensure that the hardware configuration is correct by double-clicking on "Hardware Configuration" in the left-hand menu. There should be a green arrow next to "LCMS System". If there is not, click once on "LCMS System" and then select "Activate Profile".

11.4.8 In the Project dropdown menu, select the appropriate project. Currently, all data, methods, results, etc., concerning acrylamide are stored in the project "Hydraulic Fracturing\Acrylamide".

11.4.9 Ensure that LC solvent levels are adequate and that there is enough N_2 gas to complete the runs, and that all regulators on compressed tanks or Liquid nitrogen tanks are open.

11.4.10 Load samples into the Shimadzu SIL-20AC autosampler. In the Analyst 1.5.2 software, select Build Acquisition Batch under "Acquire" in the left-hand menu to build a new sample batch. From the dropdown menu next to the Method Editor button, select "AA_20120621.dam" to load the correct instrument parameters. Click "Add Set", and then "Add Samples". Enter an appropriate sample prefix name and a data file prefix, and type in the number of samples to be added, then click "OK". Right-click on the sample batch table, and select "Hide/Show Column". Place a checkmark next to "Sample ID", and click "OK". In the sample batch, enter appropriate sample IDs, vial numbers, and injection volumes. In the "Quantitation" tab, enter the correct concentrations of the calibration standards. Click on the "Submit" tab, and then select the "Submit" button. This action uploads the samples into the Acquisition Queue.

11.4.11 Select "View->Sample Queue" to progress to the sample queue page. Select "Acquire->Equilibrate". Select the appropriate acquisition method, enter a time of 30 min, and click "OK". This equilibrates the MS and LC conditions for a set amount of time (30 min).

11.4.12 Once the instrument is ready after equilibration (under Queue Server, the icon will say "Ready"), select "Acquire"->Start Sample". This begins the sample acquisition process.

11.4.13 The instrument will automatically go into "Standby" mode after the sample queue completes.

11.5 Data Analysis

11.5.1 In the Analyst software, select "Quantitation Wizard" under the "Quantitate" menu item in the left-hand side of the screen. Select the appropriate data files, and add them to the right-hand "Selected Samples" window. Click "Next" twice, and then choose an appropriate quantitation method to use (e.g., "AA_quant)20120718"). Click "Finish".

11.5.2 After running the Quantitation Wizard, inspect each chromatographic peak to ensure that the peak has been integrated properly.

11.5.3 Identify and confirm the presence of target analytes in the samples by

matching the expected retention times of the MRMs. The retention time window of the analytes must be within 10% of the retention time of the analyte in the midpoint calibration standard.

11.5.4 Quantitate the amounts of each analyte using the isotope dilution technique. By adding a known amount of a labeled compound to every sample prior to extraction, correction for recovery of the native analog of that compound can be made because the native compound and its labeled analog exhibit similar chemical properties upon extraction, concentration, and chromatography.

11.5.4.1 Compute the concentration of each native compound that has a labeled analog in the extract using the RR from the calibration data (section 10.6) and following equation:

$$\%\text{øë}\ \mathbf{L}\ \frac{\text{º}_{\text{Ù}}\ ¼_{\text{x}}}{\text{º}_{\text{x}}\text{ËË}}$$

Where: C_{ex}= concentration of compound in the extract, and the other terms are defined in section 10.6.

11.5.5 Calculate the extraction recovery based on the recovery of AA-d_3 using the RF from the calibration data (section 10.7) and the following equation:

$$\%\text{øë}\ \mathbf{L}\ \frac{\text{º}_{\text{Ù}}\ ¼_{\text{ôþ}}}{\text{º}_{\text{ôþ}}\text{Ë}_{\text{¿}}}$$

11.5.5.1 Using the concentration in the extract determined above, calculate the percent recovery of the labeled compounds using the following equation:

$$\%R = 100\% \times \frac{C_m}{C_{srm}}$$

Where:
%R = percent recovery of labeled analog
C_m= measured concentration of standard reference material
C_{srm} = actual spiked concentration of standard reference material

Note: During calculations, take into account the concentration factor from the 500 mL sample down to 0.5 mL following extraction/concentration.

11.5.6 Calculate the spike recoveries from the fortified blanks and fortified matrices:

$$\%R = 100\% \times \frac{(S - U)}{C_{sa}}$$

Where:
%R = percent recovery
S = measured concentration in spiked aliquot
U = measured concentration in unspiked aliquot
C_{sa} = actual concentration of spike added

11.5.7 For duplicates, the precision will be indicated by the Relative Percent Difference (RPD), to be calculated as follows:

$$RPD = \frac{(C_1 - C_2) \times 100\%}{(C_1 + C_2)/2}$$

Where:
RPD = relative percent difference
C_1 = larger of the two observed values
C_2 = smaller of the two observed values

11.5.8 If precision is calculated from three or more replicates, use %RSD rather than RPD:

$$\%RSD = (s/\bar{x}) \times 100\%$$

Where:
%RSD = relative standard deviation
s = standard deviation
$\bar{x}$ = mean of replicate analyses

12.0 Method Performance

12.1 Method performance is evaluated based on the criteria in Table 1.

12.2 Using this method, MDLs have been determined for AA to be 20 ng/L.

12.3 Extraction recovery of AA typically ranges from 20-50%.

13.0 References

Lucentini, L. et al., Determination of Low-Level Acrylamide in Drinking Water by Liquid Chromatography/Tandem Mass Spectrometry, J. AOAC Int., 92, 2009, 263-270.

EPA Method 1694. "Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS", 2007.